14. G. Meyers, A. B. Rabson, S. F. Josephs, T. Smith, F. Wong-Staal, Eds., *Human Retroviruses and AIDS 1988* (Los Alamos National Laboratory, Los Alamos, NM, 1988).
15. G. Franchini *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 2433 (1989).
16. M. Fukasawa *et al.*, *Nature* **333**, 457 (1988).
17. G. Franchini *et al.*, *AIDS Res. Hum. Retroviruses* **3**, 2 (1987).
18. T. A. Kunkel, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 488 (1985).
19. We thank P. Johnson for the SE (pA194) construct, P. Kanki for the SIV_{mac} serum, and R. C. Gallo and B. Moss for support. M.B. is a fellow of the Dutch Society for the Advancement of Pure Research (ZWO).

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Calicheamicin γ_1^I and DNA: Molecular Recognition Process Responsible for Site-Specificity

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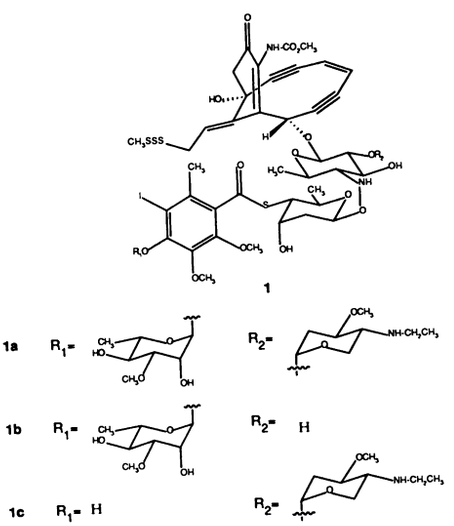
Calicheamicin γ_1^I is a recently discovered diyne-ene–containing antitumor antibiotic that cleaves DNA in a double-stranded fashion, a rarity among drugs, at specific sequences. It is proposed that the cutting specificity is due to a combination of the complementarity of the diyne-ene portion of the aglycone with DNA secondary structures and stabilization by association of the thiobenzoate-carbohydrate tail with the minor groove.

AN IMPORTANT AND INTRIGUING aspect of the phenomenally potent antitumor agent calicheamicin γ_1^I (structure **1a**) (*1, 2*) is its interaction in vitro



with DNA in which the drug makes double-stranded cuts at precise sequences (*3*). The DNA cleaving properties are believed to be initiated by the formation of a 1,4-diyl (*p*-benzyne) intermediate (structure **2**). The unexpected site-specificity of this chemistry is far from obvious, since a similar and equally potent compound, esperamicin (structure **3**) (*4, 5*), does not exhibit any strong sequence-specificity (*6*). Experimental evidence, in conjunction with molecular modeling studies reported here, provides insight into the calicheamicin-DNA encounter and may explain the distinctive sequence-specificity of such a small compound.

In order to determine the near-neighbor effect on the preferred sites TCCT/AGGA (*3*), a series of synthetic dodecamers were prepared with the tetramer sequence TCCT located at both the 5′ and 3′ ends as well as in the middle of the oligomers. Only the dodecamers having the TCCT sequence present in the middle and at the 3′ end were cut by **1a**, whereas the oligomer with the TCCT site placed at the 5′ end was not cut under these conditions (Fig. 1). The addition of a hanging sequence on either strand of the uncut dodecamer ending with 5′TCCT/3′AGGA does not facilitate or amplify cleavage (Fig. 1). This result indicates that calicheamicin γ_1^I anchors itself on the DNA from the 5′ side of the TCCT sequence (3′ side of the complementary AGGA sequence) and that **1a** requires double-stranded helical DNA in close proximity to the target site in a manner reminiscent of a restriction endonuclease.

Only the TCCT/AGGA site was subject to drug-induced cleavage and therefore this oligonucleotide-based system mimics the calicheamicin-polynucleotide restriction fragment studies (*3*). To gain further insight as

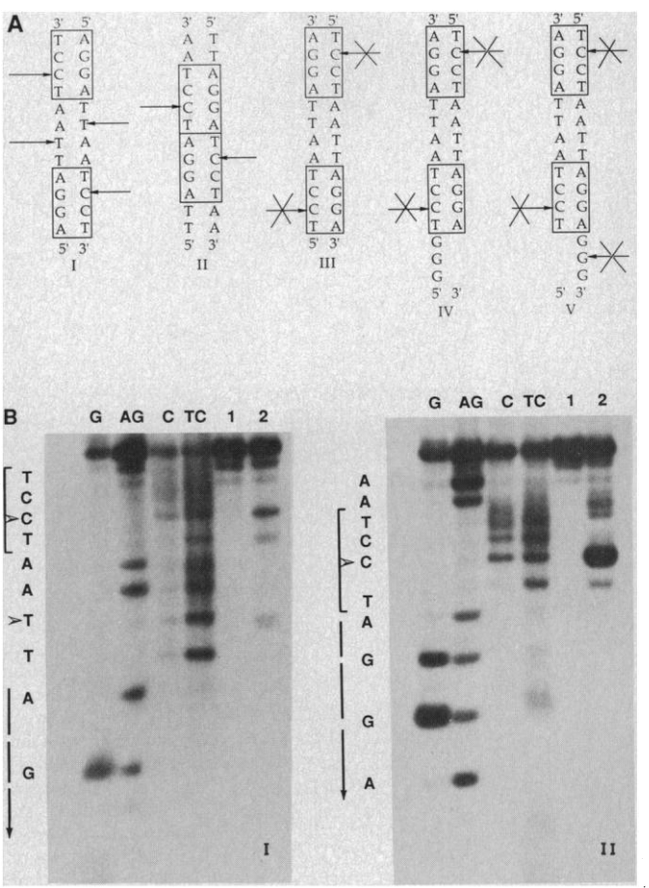


Fig. 1. (**A**) Calicheamicin γ_1^I double-strand cleavage sites on several synthetic oligomers with TCCT/AGGA sequences placed at different positions. The arrows indicate the calicheamicin γ_1^I sites of attack. (**B**) Autoradiograms of high-resolution denaturing gels of calicheamicin γ_1^I cleavage of 5′ end-labeled dodecamers I and II. Reaction conditions were 10:90 ethanol:50 m*M* tris-HCl, *p*H 7.5, carrier calf thymus DNA at 50 μg/ml [20*M* excess (in base pairs) to the drug], calicheamicin γ_1^I at 5 μg/ml, 10 m*M* β-mercaptoethanol, and end-labeled oligomer (~6000 cpm) in a total volume of 10 μl. Reactions were run for 2 hours at 4°C, then lyophilized. Cleavage products were analyzed on a 20% polyacrylamide sequencing gel at 2000 volts for 75 min. G, AG, C, TC are Maxam-Gilbert chemical sequencing lanes. Lanes 1, DNA controls; lanes 2, reactions with calicheamicin γ_1^I.

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to the significance of the TCCT/AGGA "hot" site, base pair mismatches were introduced within that tetramer sequence. An AG mismatch 5′TCAT/3′AGGA toward the 3′ side of the TCCT site does not affect the double-strand cutting. However, an AG mispair 5′TACT/3′AGGA toward the 5′ side of TCCT, that is, the same side from which the drug anchors onto the DNA, inhibits the cleaving on the AGGA-containing strand, while keeping the cut at the 5′A of the TACT-complementary tetramer (Fig. 2). An AG mispair is thought not to induce a significant deformation in the overall conformation of the DNA helix other than to produce a local widening of the minor groove (*7, 8*). The above findings suggest that the drug-reactive moiety gets partially inserted in the groove closer to the target site on the TCCT-containing strand. This conclusion is further substantiated by the observation that in some cases the cut on the AGGA side is weaker than that on the TCCT strand (Fig. 1B, part I). Furthermore, the cutting moiety of calicheamicin γ_1^I seems to be introduced in the groove at a set angle that is most probably determined by the thiobenzoate-carbohydrate tail of the drug. This effect would explain why the reactive species does not move around in the groove to a position where it could reach both strands in order to accommodate the minor deformation caused by the AG mispair. Since local helix parameters are influenced by base sequence (*9, 10*), this effect would also imply that there must exist a spatial compatibility between the cutting moiety of the drug and the complementary cleavage sites on the DNA for a double-strand scission to occur.

Creating a TC mismatch (Fig. 2) within that same sequence, 5′TCCT/3′AGTA and 5′TCCT/3′ATGA, totally inhibits the scission of either duplex strand whether the mispair is toward the 5′ or the 3′ side. TC mismatches are among the more unstable mispairs and could lead to considerable broadening of the minor groove at that site (*11*). In this case, the diyne-ene moiety finds itself in an expanded groove and hence is inaccessible to either of the DNA strands. This result confirms the earlier idea of the drug being presented in the groove at a well-determined angle. The above findings, along with initial observations (*3*) and bias of calicheamicin γ_1^I toward (G + A) · (T + C) sequences, imply that such sequences meet the spatial requirements imposed by a combination of the diyne-ene moiety and the rest of the drug molecule.

Insight as to the importance of various structural features in the carbohydrate-tail portion of calicheamicin γ_1^I for cleaving efficiency and specificity was gained from examination of the DNA cutting by certain derivatives of calicheamicin γ_1^I. The derivative lacking the 4-ethylamino sugar (structure **1b**) exhibited a cutting pattern identical to that of the parent compound but was two to three orders of magnitude less efficient. Acetylation of the 4-ethylamino sugar also lowered the cleavage affinity but not the reaction specificity. Thus, the basicity of the 4-ethylamino sugar is important in the cleaving efficiency, most likely because of its catalytic role in the activation of the trisulfide group (*12*). In addition, drug-DNA association might help bring these two groups closer to each other. However, this sugar plays no role in determining DNA cutting specificity. A derivative lacking the terminal rhamnose (structure **1c**) had the same specificity as calicheamicin γ_1^I but exhibited 50 to 100 times less cutting efficiency. Removing both the rhamnose and the 4-ethylamino sugar (structure **1** where R_1 and R_2 = H) resulted in the inhibition of cutting. So far, we have been unable to cleave selectively the glycosylated N-O bond in order to test the aglycone-disaccharide portion.

These structure-activity relations, along with the specificity of cutting at the TCCT/AGGA tetramer seeming to be independent of the nature of the flanking sequences, suggest that the carbohydrate tail–DNA interaction is a necessary but nonspecific one. Circular dichroism studies on the binding of the inactive aromatic derivative (structure **2**) (*13*) with sonicated calf thymus

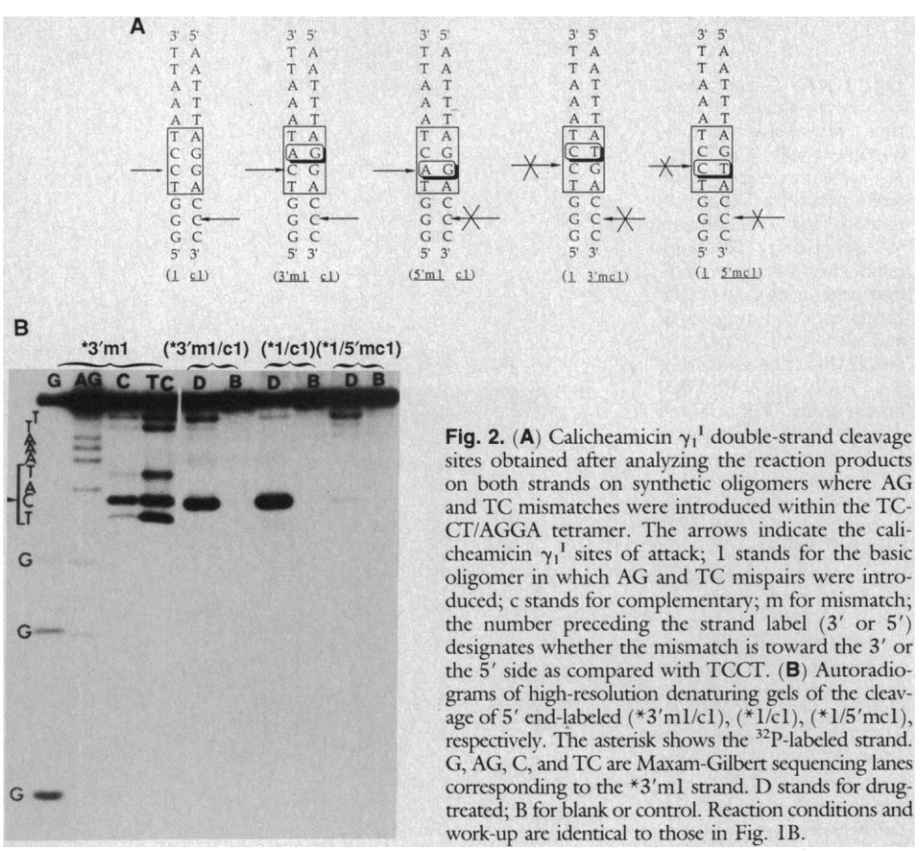
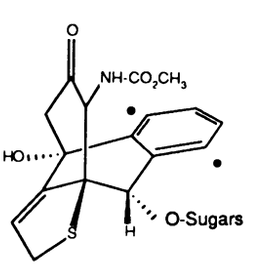


Fig. 2. (**A**) Calicheamicin γ_1^I double-strand cleavage sites obtained after analyzing the reaction products on both strands on synthetic oligomers where AG and TC mismatches were introduced within the TCCT/AGGA tetramer. The arrows indicate the calicheamicin γ_1^I sites of attack; 1 stands for the basic oligomer in which AG and TC mispairs were introduced; c stands for complementary; m for mismatch; the number preceding the strand label (3′ or 5′) designates whether the mismatch is toward the 3′ or the 5′ side as compared with TCCT. (**B**) Autoradiograms of high-resolution denaturing gels of the cleavage of 5′ end-labeled (*3′m1/c1), (*1/c1), (*1/5′mc1), respectively. The asterisk shows the ^{32}P-labeled strand. G, AG, C, and TC are Maxam-Gilbert sequencing lanes corresponding to the *3′m1 strand. D stands for drug-treated; B for blank or control. Reaction conditions and work-up are identical to those in Fig. 1B.



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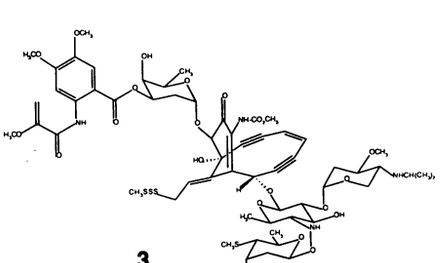
DNA showed reduction in the DNA dichroic absorption, primarily of the positive 270-nm peak. This result suggests that a tightening of the DNA duplex occurs probably



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through association between the carbohydrate tail of the drug and DNA. This association must occur in the minor groove since

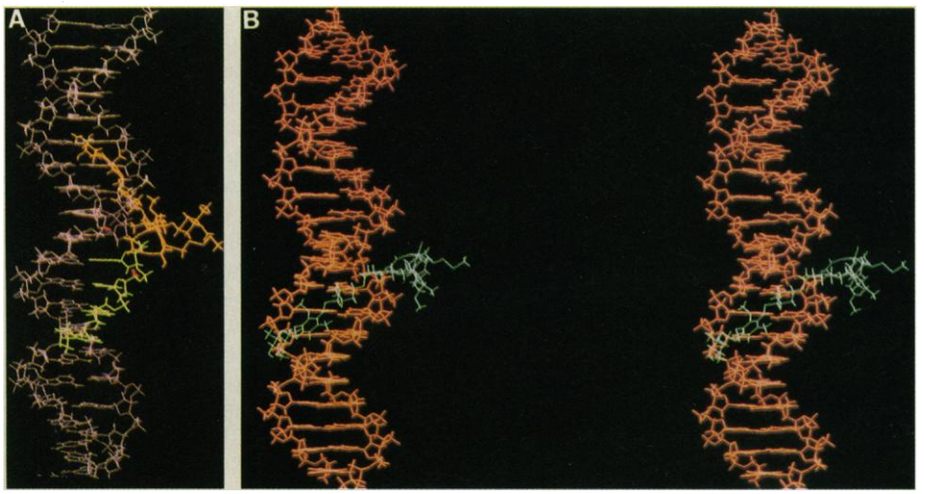

Fig. 3. Computer-generated depiction of the proposed association of calicheamicin γ_1^I with the minor groove of standard B-DNA. (**A**) The drug is in yellow, the TCCT/AGGA sequence is in green, and the hydrogens abstracted by the *p*-benzyne diradical are in red. (**B**) Stereo pair view of the carbohydrate-thiobenzoate tail of the drug (in blue) wrapped around the DNA minor groove. This tail serves as a vehicle for the entry of the diyne-ene into the DNA.

netropsin, a known minor groove binder, alters the specificity of calicheamicin γ_1^I (*3*). That calicheamicin γ_1^I cuts T_4 phage DNA (which is glycosylated in the major groove), with the same efficiency as regular DNA further supports the implication of the minor groove as the binding site.

These results lead us to propose a model for a calicheamicin γ_1^I–DNA encounter based on computer-assisted molecular modeling studies (Fig. 3) (*14*). In this depiction, the carbohydrate-thiobenzoate tail is placed in the minor groove, where the 3-hydroxyl of the thiosugar and the 2-hydroxyl of the rhamnose face the bottom of the groove and can hydrogen bond to the O-2 of juxtaposed pyrimidine bases. Additional stabilization occurs by the quasi-intercalation (*15*) of the thiobenzoate ring between the walls of the minor groove. The shape and the size of the carbohydrate-thiobenzoate tail of the molecule (Fig. 3) permit orientation along the minor groove of the DNA (in a 3′ purine · 5′ pyrimidine fashion) by equilibrium binding due to the above-mentioned contacts. This association then guides the diyne-ene cutting moiety into the duplex DNA groove at sites where the local helix characteristics (for example, groove width and angles) are favorable for such an insertion (Fig. 3). One must bear in mind that the movement of the diyne-ene moiety within the groove is then controlled by the carbohydrate-thiobenzoate tail of the calicheamicin γ_1^I molecule. In the presence of thiols, the diradical species then forms in the groove. At sites such as TCCT/AGGA, where the spatial orientations in the groove are optimal, the diradical abstracts the hydrogens it requires from the sugars, which initiates DNA double-strand scission (the most favored hydrogens being in this case the 5′H on the TCCT strand and the 4′H two bases off toward the 3′ side on the AGGA-containing strand) (Fig. 3) (*16*). However, if the diradical forms in a site on the DNA that is "too wide" and where the hydrogens on the deoxyribose sugars are not accessible then it abstracts hydrogens from only one strand (as in a case of a mismatch) or from the thiol-solvent system (as in the case of unfavored sites) to form the inactive aromatic derivative (structure **2**). This situation would explain the results of Fig. 2. In contrast, esperamicin (*4, 5*) might not be able to associate with the DNA as closely as calicheamicin γ_1^I, since it lacks the thiobenzoate-rhamnoside moiety and is therefore less specific. Moreover, experamicin might have more than one choice in forming a complex with the DNA, given that it possesses two different sets of groupings hanging off its diyne-ene moiety.

Calicheamicin γ_1^I represents a new kind of DNA cleaving agent in which the cleavage specificity of the *p*-benzyne diradical is proposed to be directed by a nonspecific but stabilizing DNA minor groove carbohydrate-aromatic interaction, a rarity among drugs. The potent biological activity of calicheamicin γ_1^I could be due to its ability to cause double-strand cleavage of the genetic material at specific sites that are at or adjacent to biologically important sequences on the DNA.

Note added in proof: On the basis of our initial studies on DNA restriction fragments (*3*) another group (*17*) recently proposed a binding model for calicheamicin γ_1^I. In this model, it is suggested that the drug anchors onto the DNA from the 5′ side of AGGA and abstracts a C-1′ hydrogen from the AGGA-containing strand. This is unlikely in light of our orientation studies described herein as well as our observations on electrophoretic mobilities (*16*) in the case of AGGA strand cleavage.

REFERENCES AND NOTES

1. M. D. Lee *et al.*, *J. Am. Chem. Soc.* **109**, 3464 (1987).
2. M. D. Lee *et al.*, *ibid.*, p. 3466. The stereochemistry at C-8 has been reassigned to the α epimer based on the nuclear magnetic resonance (NMR) of some synthetic model compounds. See A. S. Kende and C. A. Smith, *Tetrahedron Lett.* **29**, 4217 (1988).
3. N. Zein, A. M. Sinha, W. J. McGahren, G. A. Ellestad, *Science* **240**, 1198 (1988).
4. J. Golik *et al.*, *J. Am. Chem. Soc.* **109**, 3461 (1987).
5. J. Golik *et al.*, *ibid.*, p. 3462.
6. B. H. Long *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 2 (1989); Y. Sugiura, Y. Takahashi, Y. Uesawa, J. Kuwahara, *Nucleic Acids Res.*, Symposium Series No. 20 (IRL Press, Oxford, 1988), p. 63.
7. G. G. Privé *et al.*, in *Structure and Expression*, M. H. Sarma and R. H. Sarma, Eds. (Adenine, Schenectady, NY, 1988), vol. 2, pp. 27–48; G. G. Privé *et al.*, *Science* **238**, 498 (1987).
8. W. N. Hunter, T. Brown, N. N. Anand, O. Kennard, *Nature* **320**, 552 (1986).
9. H. Wing *et al.*, *ibid.* **287**, 755 (1980); A. Fratini, M. Kopka, H. Drew, R. Dickerson, *J. Biol. Chem.* **257**, 14686 (1982); H. Nelson, J. Finch, B. Luisi, A. Klug, *Nature* **330**, 221 (1987); W. Hunter *et al.*, *J. Biol. Chem.* **262**, 9962 (1987); M. McGall, T. Brown, W. N. Hunter, O. Kennard, *Nature* **322**, 661 (1986).
10. M. Nilges, G. M. Clore, A. M. Gronenborn, N. Piel, L. W. McLaughlin, *Biochemistry* **26**, 3734 (1987). R. D. Wells, *J. Biol. Chem.* **263**, 1095 (1988); D. A. Pulleyblank, D. B. Haniford, A. R. Morgan, *Cell* **42**, 271 (1985); T. Evans and A. Efstratiadis, *J. Biol. Chem.* **261**, 14771 (1986); T. Kohwi-Shigematsu and Y. Kohwi, *Cell* **43**, 199 (1985).
11. P. Modrich, *Annu. Rev. Biochem.* **56**, 435 (1987).
12. G. A. Ellestad *et al.*, unpublished data.
13. The aromatic derivative (structure **2**), in which only the aglycone was altered, possesses a very weak dichroism.
14. The MOGLI graphics package was used on an Evans and Sutherland PS530 system to manually dock the drug onto the DNA molecule.
15. C. Yoon, G. G. Prive, D. S. Goodsell, R. Dickerson, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 6332 (1988).
16. Evidence for abstraction of a 5′-hydrogen on the TCCT strand has been previously reported (*3*). Unpublished results from these laboratories (present authors) suggest that on the complementary strand a 4′-hydrogen is abstracted. This result is based on electrophoretic mobilities of DNA fragments resulting from cleavage of 5′ end-labeled dodecamers containing the AGGA site. These fragments migrate slightly ahead of the chemically produced markers suggestive of 3′ termini ending in phosphoglycolate moieties similar to observations with bleomycin [L. Giloni, M. Takeshita, F. Johnson, C. Inden, A. P. Grollman, *J. Biol. Chem.* **256**, 8608 (1981)].
17. R. C. Hawley, L. L. Kiessling, S. L. Schreiber, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 1105 (1989).

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